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# Persistence and survival of *Cryptosporidium parvum* oocysts on lamb's lettuce leaves during plant growth and in washing conditions of minimally-processed salads

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ABSTRACT

*Cryptosporidium* is the causative agent of cryptosporidiosis, which results, among others, in profuse diarrhoea. Transmission to humans occurs via the faecal-oral route directly by contact with infected hosts or indirectly by waterborne or foodborne routes. For the latter, parasite transmission is closely linked to the oocyst's ability to persist and survive in food matrices. In this study, we evaluated the persistence and survival of *Cryptosporidium* oocysts in lamb's lettuce: i) during plant growth and ii) in conditions mimicking the industrial washing process applied in minimally-processed vegetables (MPV). Results show that oocysts persisted during the growth of lamb's lettuce, i.e. two months from the 2-leaf stage until the 8-leaf harvest time (−0.89 Log10 of oocysts). However, their survival decreased from as early as one week (−0.61 Log10), and only 6 % of oocysts remained infective at the time of harvest. The washing process had a limited effect on parasite load (<0.5 Log10) and no effect on survival; chlorination of washing water did not improve the efficiency (removal and inactivation) of the process. The ability of *C. parvum* to persist and survive throughout the food chain may drive its transmission to humans through MPV products. Appropriate management measures should be implemented at each operational level to limit contamination and ensure food safety of fresh produce.

## 1. Introduction

Over the last decades, annual outbreak surveillance has shown a rise in the number and size of foodborne outbreaks of cryptosporidiosis disease (World Health Organization, 2015). In 2019 in Europe, 5175 foodborne outbreaks, including waterborne outbreaks, were reported, of which 0.6 % were due to parasites (EFSA and ECDC, 2021). Among parasites, the protozoan *Cryptosporidium* was responsible for 11/31 outbreaks and 468 cases. Interestingly, Nordic countries still account for most of the reports as previously described (Robertson and Chalmers, 2013). At the global level, in 2010, *Cryptosporidium* was responsible for 8.6 million cases of foodborne illnesses, 663,759 deaths and 296,156 disability-adjusted life years (Ryan et al., 2018). However, the number of cases of foodborne cryptosporidiosis remains under-reported (EFSA Panel on Biological Hazards et al., 2018). Indeed, the long incubation period (≈7 days) renders it difficult: i) to characterise the outbreaks, ii) to identify the food origin, and iii) to retrieve appropriate samples for testing. Furthermore, techniques for recovering and detecting *Cryptosporidium* oocysts from different food matrices are challenging (Barlaam et al., 2022; Berrouch et al., 2022; Chalmers et al., 2020; Trelis et al., 2022). For foodborne cryptosporidiosis, fruits and vegetables, and more particularly leafy greens consumed raw or minimally processed, account for most of the cases (EFSA and ECDC 2021; Nasser, 2022). Vermeulen et al. (2017) estimated a total global *Cryptosporidium* load from livestock manure of $3 \cdot 10^{23}$ oocysts/year, with the largest contributors being cattle (calves), followed by chickens and pigs. Consistent with this, the relationship between *Cryptosporidium* in livestock, and water, soil and fresh produce contamination has been highlighted (Bordes et al., 2020;

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Nasser, 2022; Rzezutka et al., 2010). Furthermore, food processing, including washing with contaminated water, and food handling, due to poor hygiene practices of infected food handlers, can also be responsible for fresh produce contamination (EFSA Panel on Biological Hazards et al., 2018).

Transmission to – and infection of – humans through ingestion of contaminated fresh produce occur if *Cryptosporidium* oocysts persist (i.e. remain durably in food) and survive (i.e. keep their infectious potential). This means that *Cryptosporidium* oocysts must withstand environmental stress conditions (e.g. desiccation, extreme temperatures, UV irradiation) during primary production (i.e. plant growth) and/or industrial processes, depending on the contamination point. It has been demonstrated that *Cryptosporidium* oocysts can survive up to months in environmental waters, depending on the type of water and the temperature, while drying and UV reduced their survival (Gérard et al., 2019; Pokorny et al., 2002; Robertson et al., 1992). In minimally processed vegetable (MPV) industries, fresh produce is washed in tap water, with or without chemicals, under continuous mechanical agitation, usually ensured by air bubbling. This washing step aims at reducing the microbial load naturally present on the surface of produce, from 0.5 to 2 Log10 (Julien-Javaux et al., 2019). Depending on the country and in agreement with national regulations, chemical disinfectants can be added to washing water. The objectives are to improve the efficacy of washing by inactivating the microorganisms and to avoid cross-contamination between batches during washing by maintaining microbiological water quality (Banach et al., 2015). Among chemicals, chlorine is the most widely used disinfectant, mainly because it has a broad-spectrum antibacterial activity, good solubility and stability in water, is easy to handle, and is cost effective. Chlorine is usually applied at concentrations ranging from 50 to 200 mg/L for 30 s to 2 min (CT, i.e. concentration time, from 25 to 400 mg·min/L) and at pH $< 8.0$ and low temperatures (5–8 °C) (Goodburn and Wallace, 2013). Although these CTs are efficient against bacteria, the literature shows that they are inefficient at inactivating *Cryptosporidium* oocysts in aqueous solutions (Gérard et al., 2019). However, the interaction of pathogens with foodstuffs can modify their persistence and/or survival to different stresses, either in a positive (protection) or in a negative way (enhanced inactivation) (De Baerdemaeker et al., 2022; Deboosere et al., 2004; Kingsley and Chen, 2009; Li et al., 2016; Su et al., 2010).

This study aimed to document and assess the persistence and survival of *C. parvum* oocysts on lamb's lettuce leaves during plant growth and during washing in conditions mimicking the industrial process with or without disinfectant (i.e. chlorine). With a high probability of contamination (growth close to the ground) and subjected to gentle washing due to its fragility and being consumed entirely raw, lamb's lettuce has been chosen as the model of leafy green. The newly produced data will contribute to a better understanding of the transmission of human cryptosporidiosis through the food route and help to implement efficient control measures.

## 2. Materials and methods

The chemicals and reagents used in this present study are given in Appendix (Table 1).

### 2.1. Cryptosporidium parvum oocysts and purification

*Cryptosporidium parvum* oocysts were purchased at the National Institute of Agricultural, Food and Environmental Research (INRAE), Nouzilly, France. The isolate was initially sampled from an infected child and maintained in the laboratory by serial passage on calves at INRAE. Oocysts from faeces of experimentally infected calves were concentrated and purified as previously described (Kubina et al., 2021). Purified oocysts were stored at +4 °C in phosphate-buffered saline solution (PBS, Gibco™, Thermo Fisher Scientific, France) until use. Fresh oocysts (less than three months old) were used in the contamination experiments.

### 2.2. Lamb's lettuce

In this study, a winter salad was used, the lamb's lettuce "GALA", which is the most common variety cultivated in France. Seeds were purchased from a specialised retailer (Hubert, France) and placed in small (80 mm diameter) plastic cups filled with universal compost (Truffaut, France) with drainage holes in the bottom. For optimum conditions for germination, seeds were buried at 0.5 cm, as described elsewhere (Ahmed et al., 2017; Wu et al., 2018). Seeding was performed at room temperature with daily irrigation and exposure to natural light. Once germination occurred, the plants were maintained at room temperature until the 2-leaf stage.

Commercially available packages of Lamb's lettuce labelled "rinse before serving" were also used and purchased in local supermarkets. Before use, the absence of *Cryptosporidium* DNA in the salads was controlled by real-time qPCR (see below).

### 2.3. Experimental contamination and plant growth conditions of lamb's lettuce

Lamb's lettuce plants at the 2-leaf stage (see above) were contaminated with *C. parvum* oocysts ($10^5$) on the upper surface of the leaves (5 droplets of 1 µL) to mimic contamination through sprinkler irrigation. Then plants were directly incubated in a temperature-controlled chamber corresponding to winter cultivation conditions: +10 ± 0.27 °C with humidity at 95 ± 5.51 % and a short day of light exposure (10/24 h; special LED growth, full spectrum; Agrolight, Hydrozone, France). Irrigation of plants was carried out by roots once a week until the harvest stage. The plants were allowed to grow for two months, corresponding to the 8-leaf stage, which is the harvest stage in primary production. Plants ($n \geq 2$) were collected weekly from Day 1 to Day 57 and analysed to evaluate oocyst persistence and survival as described below (Fig. 1A, C). For each experiment and time-point, non-inoculated plants were prepared and analysed as negative controls. At least three independent growth experiments were performed.

### 2.4. Experimental contamination and washing conditions of lamb's lettuce

Lamb's lettuce from commercial packages (30 g) was placed in a Stomacher bag (Interscience, France) and contaminated with 100 µL of suspension ($10^4$ oocysts) distributed across different areas of the sample in ten droplets (Chalmers et al., 2020). After a contact time of 3 days at +4 °C, contaminated salads were washed for 30 s under agitation (bubbling; Aqua Store, France) in 3 L of Milli-Q water (Merck Millipore, Germany; ratio product/water = 0.01) supplemented (or not) with chlorine at a final concentration of 60 mg/L (pH = 6) (3.81 mL of chlorine 4.735 %; AcrosOrganics, Thermo Fisher Scientific, Denmark). These washing conditions correspond to a CT (concentration × time) of 30 mg/L·min commonly used in French minimally-processed vegetable industries. Salads were then rinsed by soaking in pure water (Milli-Q), spun in a salad spinner (20 spins/10-s break/10 spins) and finally placed in a new sterile Stomacher bag to be analysed for oocyst persistence and survival (see below and Fig. 1B, C). Samples of contaminated salads were directly analysed to determine the initial number of total and infective oocyst equivalents before the process (N0 starting oocyst number). Three samples per experiment and at least three independent experiments were performed.

### 2.5. Recovery of C. parvum oocysts from lamb's lettuce to evaluate oocyst persistence and survival

*C. parvum* oocysts were recovered from 30 g samples as previously recommended for optimal recovery (Robertson and Gjerde, 2001).

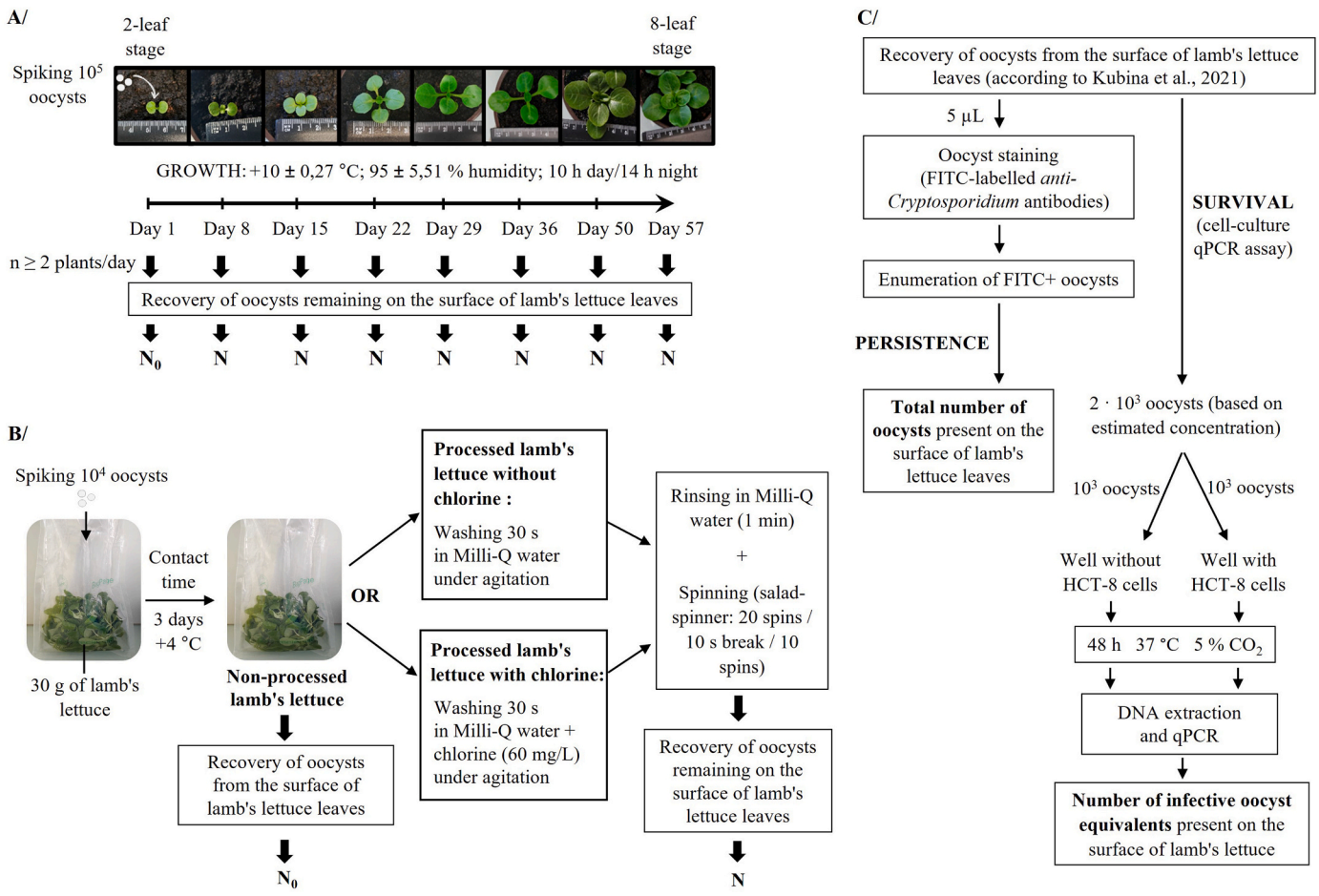


**Fig. 1.** Experimental designs to assess *C. parvum* oocysts persistence and survival in lamb's lettuce during plant growth conditions (A) and washing conditions (B). Workflow for persistence and survival analyses (C).

Recovery was performed according to Kubina et al. (2021). In the case of contaminated plants (from the 2-leaf stage to the 8-leaf stage), *Cryptosporidium*-free commercial lamb's lettuce was added to reach a 30 g sample. Briefly: oocysts were eluted with 180 mL glycine 1 M pH 3.0 in a paddle homogeniser (BagMixer, Interscience, France) for 1 min (speed eight strokes/s). After washing with PBS, parasites were concentrated by centrifugation (3000 *g* for 30 min at +4 °C) and purified using immunomagnetic separation (IMS, Isolate for IMS of *Cryptosporidium* oocysts, TCS Biosciences, UK).

### 2.6. Enumeration of *C. parvum* oocysts and evaluation of oocyst persistence in lamb's lettuce

Five microliters from purified oocyst suspensions were deposited in slide wells (Teflon printed diagnostic slide, Immuno-cell, Belgium). Oocysts were stained with FITC-labelled anti-*Cryptosporidium* antibodies (Crypto Cel FITC; TCS Biosciences, UK) according to the manufacturer's protocol. Fluorescent oocysts (FITC+) were then counted using an epifluorescence microscope (Leitz, Diaplan) at X 400 magnification, and the concentration of oocysts/mL was determined. *C. parvum* persistence was defined as the ability of oocysts to remain on lamb's lettuce leaves in different conditions, irrespective of their infective state. The number of remaining oocysts on the surface of the leaves corresponded to the number of FITC+ oocysts recovered from lamb's lettuce in each condition and expressed as Log10.

### 2.7. Cell culture (CC)-qPCR assay for infectivity assessment

Infectivity was evaluated using a previously described *in vitro* assay based on the inoculation of HCT-8 (human ileocecal adenocarcinoma) cell cultures with oocysts (without prior excystation). HCT-8 cells (ATCC CCL-224) were maintained and prepared to obtain 90 % confluent monolayers as previously described (Kubina et al., 2021).

For each sample, oocysts recovered from lamb's lettuce were enumerated (see above and Fig. 1C) and $2 \cdot 10^3$ oocysts were resuspended in 2 mL of co-culture medium (RPMI 1640 medium (Gibco™, Fisher Scientific, France) supplemented with 45 mg/mL of glucose (Sigma, France), 40 µg/mL of para-aminobenzoic acid (Sigma, France), 40 µg/mL of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid "HEPES" (Sigma, France), 0.35 mg/mL of ascorbic acid (Sigma, France), 1 UI/mL of insulin (Sigma, France), 100 UI/mL of penicillin and 100 pg/mL streptomycin (Corning™; Thermo Fisher Scientific, France). Then 1 mL of this parasite suspension was deposited in one well with cells and 1 mL in one well without cells, i.e. theoretically $10^3$ oocysts per well (flat-bottom 24-well plate, Thermo Fisher Scientific, Denmark). After 48 h of incubation at 37 °C in a 5 % $CO_2$ atmosphere, total DNA was extracted from each well using the QIAmp DNA Mini kit (Qiagen, France). The genome of *Cryptosporidium* was detected in each DNA extract by a TaqMan qPCR assay targeting a specific 452 bp *C. parvum* sequence (GenBank accession No. AF188110; Fontaine and Guillot, 2002) using the same qPCR reaction mix as the one previously described (Kubina et al., 2021). Single plex qPCR assays were carried out with the CFX96™ thermal cycler (Bio-Rad, France), and amplification curves were analysed using BioRad CFX Manager software. The Cq values corresponding

to the quantification cycle were registered for DNA extracted from wells with (Cqw/cells) and without cells (Cqw/o cells) for each sample. A qPCR was considered positive if Cq < 40. Each DNA extract was analysed in duplicates by qPCR. CC-qPCR assays with control oocysts (i.e. from stock solution) were performed in parallel in each assay to check the infectivity of oocyst batches.

### 2.8. Establishment of standards for quantification of total and infective oocyst equivalents by CC-qPCR

First, a qPCR standard curve was produced using ten-fold dilutions of copies of a synthetic dsDNA corresponding to the AF188110 sequence (Cq = f(Log10(AF188110 copies/reaction)). Second, suspensions of different batches of freshly excreted infective oocysts were prepared, and 10,000 to 1 infective oocysts were resuspended in 1 mL of co-culture medium and deposited in wells with and without HCT-8 cells to be further processed as in the CC-qPCR assay described above. The number of copies of the target sequence AF188110 present in each well was derived from the Cq values (Cq mean, at least two qPCR replicates) obtained in the CC-qPCR assay using the qPCR standard curve. Third, dose-response calibration curves were established for wells with and without cells by plotting the Log10 (number of AF188110 copies) to the Log10 (number of infective oocysts deposited in wells with and without cells) (Appendix, Fig. 1).

For experimental samples in which the state of the oocysts (i.e. infective or not) and the quantity of infective oocysts were unknown, the curve drawn from wells without cells allowed to quantify all oocysts, irrespective of their infective state (total oocyst equivalents, $N_{tot.eq}$) and the one drawn from the wells with cells allowed to quantify infective oocysts (infective oocyst equivalents, $N_{inf.eq}$), i.e. parasites that can infect cells and multiply (Appendix, Fig. 2).

### 2.9. Quantification of infective oocyst equivalents and evaluation of oocyst survival in lamb's lettuce during growth and the washing process by CC-qPCR

qPCR was performed at least in duplicates on each DNA extract (with or without cells). Cq means were then calculated from all qPCR replicates (Cq < 40), and only Cq means below quantification thresholds of the CC-qPCR assay on lamb's lettuce were retained for quantification (i.e. Cq mean < 36 and < 37 in wells with and without cells, respectively; Kubina et al., 2021). For valid Cq values, quantification was performed in wells with and without cells using the qPCR standard curve and corresponding dose-response calibration curves (see above and Appendix, Fig. 1, 2). The number of infective oocyst equivalents at the start of experiments ($N_0$), i.e. at Day 1 for growth trials and before washing for process assays, was calculated for each sample (($N_{inf.eq}$)$_0$; wells with cells), transformed in Log10 and averaged. To determine the number of infective oocyst equivalents during growth or after the washing process (N), data were normalised as follows: 1) the number of total oocyst equivalents at the start of experiments was calculated for each sample (wells without cells; ($N_{tot.eq}$)$_0$). Then the mean number of total oocyst equivalents ($N_{tot.eq}$)$_{0\ MEAN}$ was determined for each independent experiment (X = 4 for growth experiments; X = 3 for washing process experiments). 2) for each sample during growth or at the end of the washing process, the numbers of total oocyst equivalents ($N_{tot.eq}$) and infective oocyst equivalents ($N_{inf.eq}$) were determined. 3) for each sample, a normalisation factor NF was calculated: $NF = (N_{tot.eq})_{0\ MEAN} / N_{tot.eq}$. and 4) the numbers of infective oocyst equivalents were normalised in each sample and each condition: $N_{inf.eq.norm} = N_{inf.eq} \times NF$. For each sample, results were transformed as Log10($N_{inf.eq.norm}$), and the mean Log10($N_{inf.norm}$) ± standard deviation was finally expressed for each condition based on all independent experiments (Appendix, Fig. 3).

### 2.10. Statistical analysis

Experiments were performed in replicates at least thrice. All data were transferred to R version 3.6.3 (R Foundation for Statistical Computing, Austria) software. Considering that the number of data varied (from 3 to 15), the non-parametric test of Wilcoxon was used to compare persistence and survival data. Statistical difference was considered if *P*-value <0.05.

## 3. Results

### 3.1. Persistence and survival of C. parvum oocysts on lamb's lettuce leaves during plant growth

We estimated the persistence of *C. parvum* oocysts by counting fluorescent oocysts (FITC+) recovered from salad leaves at different times of growth. The number of FITC+ oocysts significantly decreased after 2 weeks compared to Day 1 (4.16 ± 0.31 Log10 at Day 1 *vs*. 3.88 ± 0.40 Log10 at Day 15; *P*-value <0.05; Fig. 2). A further significant decrease was observed after 43 days compared to Day 15 (3.88 ± 0.40 Log10 at Day 15 *vs*. 3.48 ± 0.30 Log10 at Day 43; *P*-value <0.05; Fig. 2). Nevertheless, after two months of growth, 3.27 ± 0.25 Log10 of FITC+ oocysts were still recovered from the leaves at the time of harvest (Day 57, −0.89 Log10 compared to Day 1). The survival of *C. parvum* oocysts was significantly affected from one week of lamb's lettuce growth, with the number of infective oocyst equivalents decreasing from 3.48 ± 0.34 Log10 at Day 1 to 2.87 ± 0.79 Log10 at Day 8 (−0.61 Log10; *P*-value <0.05; Fig. 3). This reduced survival of *C. parvum* oocysts continued during the second week, as demonstrated by the significant decrease from 2.87 ± 0.79 Log10 at Day 8 to 1.86 ± 0.57 Log10 at Day 15 (*P*-value <0.05). Then from Day 15 to harvest time, the number of surviving oocysts did not significantly vary (except Day 29 *vs*. Day 43, *P*-value = 0.015), and about 6 % of infective oocysts were still present after two months of growth (Day 57, 1.95 ± 0.80 Log10; Fig. 3).

### 3.2. Impact of washing conditions on the persistence and survival of C. parvum on lamb's lettuce leaves

The washing conditions that were tested in this study consisted of a three-step process: i) washing in water, without or with chlorine at 60 mg/min (CT = 30 mg·min/L); ii) rinsing with water; and iii) spinning. Persistence and survival were evaluated at the end of these three steps.

The overall process reduced the number of oocysts recovered from the leaves, from 3.19 ± 0.08 Log10 to 2.70 ± 0.18 Log10 for washing without chlorine (*P*-value <0.001) and to 2.71 ± 0.09 Log10 for washing with chlorine (*P*-value <0.001; Fig. 4).

Chlorine did not enhance oocyst removal from the leaves (*P*-value = 0.72; a, Fig. 4).

The number of infective oocyst equivalents slightly decreased after the washing process from 3.53 ± 0.23 Log10 to 3.22 ± 0.43 Log10 (*P*-value = 0.16) after washing with chlorine and to 3.18 ± 0.33 Log10 (*P*-value <0.05) after washing without chlorine (Fig. 5). As expected, washing with chlorine did not further inactivate oocysts compared to washing without chlorine (*P*-value = 0.86; Fig. 5).

## 4. Discussion

Transmission of *Cryptosporidium* to humans via consumption of lamb's lettuce is possible if oocysts persist and survive on this matrix from farm to fork. Two studies demonstrated that *C. parvum* oocysts spiked on leafy greens (lettuce and basil leaves) remained viable under refrigerated conditions from 8 days to 2 weeks (Hohweyer et al., 2016; Utaaker et al., 2017). Using nested-PCR, another study showed that *Cryptosporidium* could persist on herbs as cilantro and parsley up to 3 days under natural environmental conditions (in the field) and at least 23 days in growth chamber conditions (Oguadinma, 2019). For the first

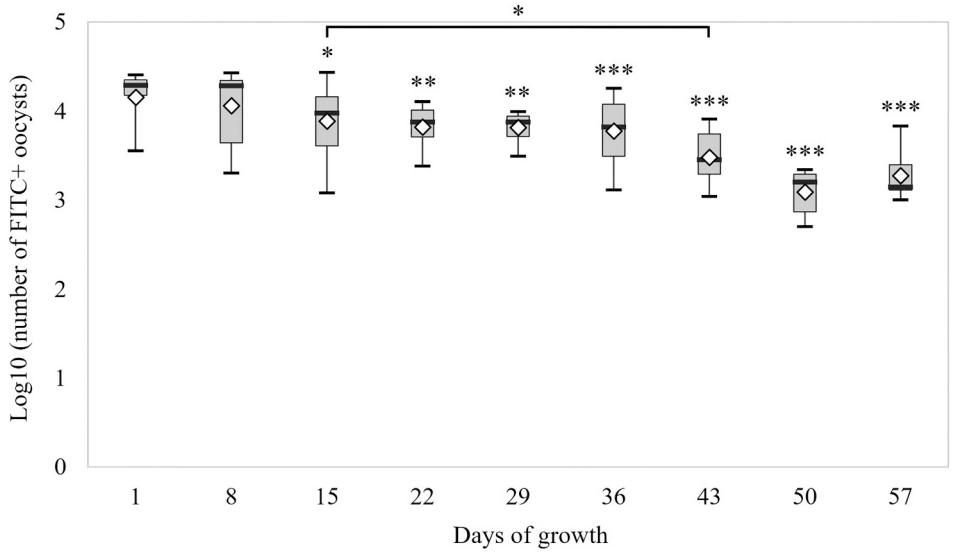


**Fig. 2.** Persistence of *C. parvum* oocysts on lamb's lettuce leaves during plant growth. At each time point, parasites were recovered from the leaves, stained with FITC and FITC+ oocysts were enumerated. Rhombs represent means. The asterisk above each box corresponds to the statistical comparison relative to Day 1. *P*-value ≤0.001 (***), ≤0.01 (**), ≤0.05 (*).

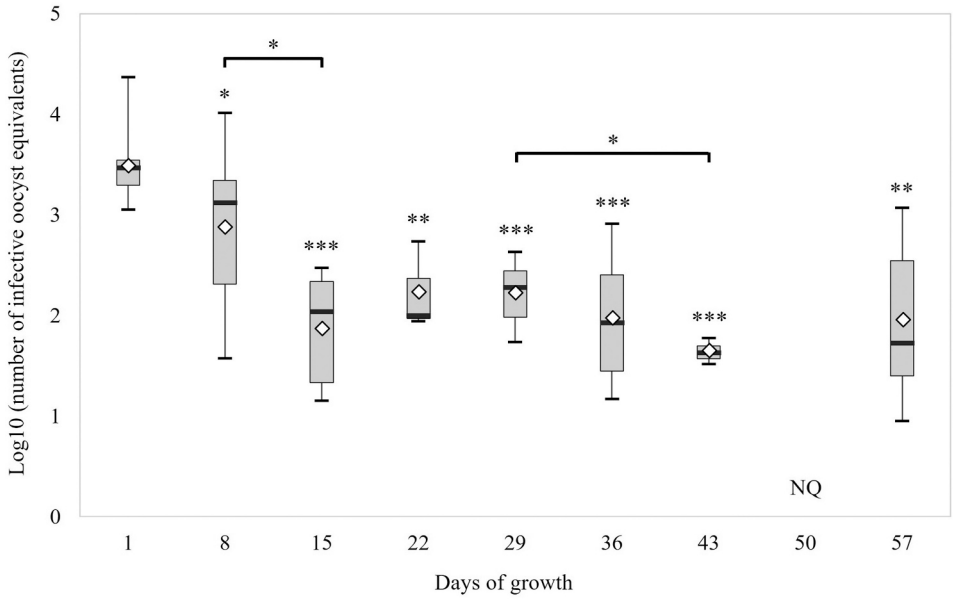


**Fig. 3.** Survival of *C. parvum* oocysts on lamb's lettuce leaves during plant growth. At each time point, the parasites were recovered from the leaves and inoculated into the cell cultures. The number of infective oocyst equivalents was determined by CC-qPCR. Rhombs represent means. NQ: no quantifiable samples. The asterisk above each box corresponds to the statistical comparison relative to Day 1. *P*-value ≤0.001 (***), ≤0.01 (**), ≤0.05 (*).

time, we explored the persistence but also the survival (i.e. infective character) of *C. parvum* oocysts on lamb's lettuce leaves during plant growth. Over the 57 days of lamb's lettuce growth in conditions mimicking winter culture, we observed a one Log10 reduction in the number of *C. parvum* oocysts on the surface of leaves (Fig. 2). However, oocysts were still present at the 8-leaf harvest stage (3.27 ± 0.25 Log10 at Day 57 *vs* 4.16 ± 0.31 Log10 at Day 1; Fig. 2), suggesting that in the tested conditions, oocysts were able to persist on the surface of lamb's lettuce leaves throughout growth. We hypothesised that the decrease in FITC+ oocyst number reflected a reduction in oocyst persistence, i.e. a loss of oocysts over time likely due to a loss of adherence. Indeed, the growth condition applied in this study may affect the oocyst structure and/or wall, inducing a change in their adherence capacity. However, rather than a loss of adherence, the diminution of the number of FITC+ oocysts recovered from the leaves observed over time (Fig. 2) could also be due to the internalisation of oocysts in the stomata of lamb's lettuce leaves as previously described for spinach (Macarisin et al., 2010), or a less efficient recovery of oocysts from the leaves. In the latter case, the alterations of the oocyst structure and/or wall induced by the culture conditions may have: (i) reinforced some interactions with the leaves and increased the adherence of oocysts, (ii) limited the efficacy of the IMS step, which is based on the recognition of oocyst surface epitopes by antibodies. However, we also observed around a one Log10 reduction in the number of oocysts over two months of growth when quantifying the *C. parvum* oocysts by qPCR (data not shown). In this case, the whole DNA of inoculated leaves was extracted without the IMS step and all oocysts could be quantified irrespective of their localisation on the leaves and of their structure. These results confirm that the decrease of FITC+ oocysts

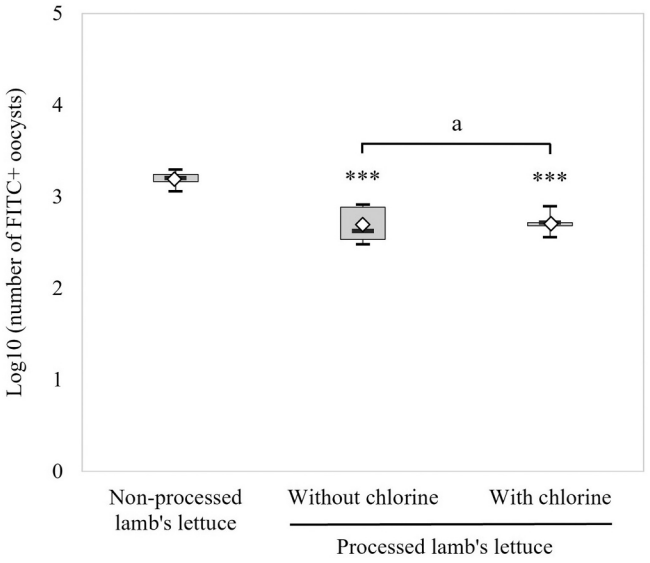


**Fig. 4.** Persistence of *C. parvum* oocysts on lamb's lettuce leaves following the washing process (i.e. washing-rinsing-spinning). Before (non-processed lamb's lettuce) and at the end of the process (processed lamb's lettuce), parasites were recovered from the leaves, stained with FITC, and FITC+ oocysts were enumerated. Rhombs represent means. The asterisk above each box corresponds to the statistical comparison relative to non-processed lamb's lettuce (*P*-value ≤0.001 (***)). (a): comparison between processed lamb's lettuce without and with chlorine (*P*-value = 0.72).

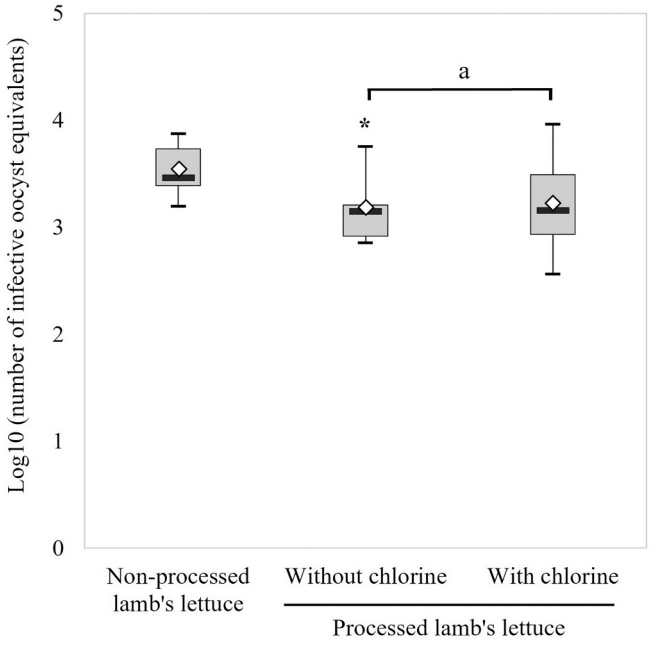


**Fig. 5.** Survival of *C. parvum* oocysts on lamb's lettuce leaves following the washing process (i.e. washing-rinsing-spinning). Before (non-processed lamb's lettuce) and at the end of the process (processed lamb's lettuce), parasites were recovered from the leaves and inoculated into the cell cultures. The number of infective oocyst equivalents was determined by CC-qPCR. Rhombs represent means. The asterisk above the box corresponds to the statistical comparison relative to non-processed lamb's lettuce (*P*-value ≤0.05 (*)). (a): comparison between processed lamb's lettuce without chlorine and with chlorine (*P*-value = 0.86).

during the growth stage of lamb's lettuce was due to a loss of parasites rather than internalisation or oocyst recovery issues. However, the origin of loss of adherence has yet to be determined. In our work, we mimicked early contamination during the growth stage of lamb's lettuce (2-leaf stage). However, the adherence of *Cryptosporidium* may be different on young and old leaves. Indeed, Esseili et al. (2011) have shown that the adherence of virus-like particles of a human *Norovirus* GII.4 strain was higher (1.5- to 2-fold) on older leaves of lettuce, probably because the carbohydrate content changed during leaf maturation. Hence, it would be interesting to test if *Cryptosporidium* oocysts persist differently on lamb's lettuce depending on the growth stage of contaminated leaves.

Survival analyses were performed using a CC-qPCR assay previously described based on the inoculation of cell cultures with unexcysted oocysts (Kubina et al., 2021). If these oocysts are infective, they can excyst and release sporozoites that can infect cells. In this case, parasite multiplication occurs, which is reflected by an increase in parasite DNA content in the presence of cells (wells with cells) but not in their absence (wells without cells), as detected by real-time qPCR. Despite the enumeration of the recovered oocysts by microscopy in each condition and the theoretical deposit of $10^3$ oocysts per well, the numbers of total oocyst equivalents inoculated in the wells slightly varied from one sample to another and from one condition to another (day of growth or process). These variations directly affected the number of infective oocyst equivalents measured in wells with cells. Hence, in these conditions, the changes in the number of infective oocyst equivalents over time of growth or following the washing process could reflect a loss of infectivity and/or simply the inoculation of a lower number of total oocysts to cell cultures. To circumvent this, the data obtained in wells with cells (number of infective oocyst equivalents) at each time of growth and after the washing process were normalised by considering the number of total oocyst equivalents inoculated in the wells without cells at the start of experiments ($N_0$; see details in materials and methods section and Appendix, Fig. 3). With such data treatment, survival analyses showed a significant loss of oocyst infectivity as early as one week from the start of growth (−0.6 Log10 between Day 1 and Day 8; Fig. 3), which became more pronounced after the second week (−1.01 Log10 between Day 8 and Day 15; Fig. 3). However, the remaining infective parasites survived until harvested, and 6 % of parasites were still infective two months after contamination (1.95 ± 0.80 Log10 at Day 57 *vs.* 3.48 ± 0.34 Log10 at Day 1; Fig. 3). Our results suggest that if contamination of lamb's lettuce occurs at the early stage of growth, oocysts can persist and remain infective on the surface of lamb's lettuce leaves until harvest stage, i.e. after two months under winter culture conditions.

In this study, the plants were grown in a 95 % humidity environment throughout the experiment. Despite this, the oocyst suspension droplets deposited onto the surface of the leaves were dry one week after inoculation. Hence, the loss of infectivity observed in our work from one week of growth could be due to desiccation to which *Cryptosporidium* oocysts are sensitive (Dawson, 2005). Such conditions can occur in fields during drought periods. Since plants were watered near the roots, it would be interesting to test whether spray-irrigation of the leaves would be more favourable to the survival of oocysts after one week. We used full spectrum leds, i.e. 400 nm to 740 nm, allowing photosynthesis which is essential for plant growth. Consequently, the effect of UV to which oocysts are exposed in primary production was not evaluated. Considering that exposure to sunlight and UV is known to inactivate *Cryptosporidium* oocysts (Soliman et al., 2018), we could anticipate that inactivation in field conditions would be even higher than that measured in our experiment. Consistent with this, Oguadinma demonstrated an increased loss of oocysts persistence on cilantro and parsley in field compared to the growth chamber conditions (Oguadinma, 2019).

The washing process was shown to have a limited effect on the removal of *C. parvum* oocysts (−0.4 Log10 units; i.e., 2.4-times reduction; Fig. 4). As expected at the applied concentration (60 mg/L for 30 s)

and as previously described for green peppers (Duhain et al., 2012), chlorine was not effective in inactivating *C. parvum* oocysts at the surface of lamb's lettuce leaves during washing (Fig. 5). In agreement with this, some studies described the presence of *Cryptosporidium* in ready-to-eat fresh produce (Barlaam et al., 2022; Caradonna et al., 2017; Dixon et al., 2013; Hajipour et al., 2021). Hence, contrarily to bacteria, the industrial washing process does not constitute a control measure to mitigate the *Cryptosporidium* hazard in leafy greens. At the opposite, reuse cycles of washing waters could contribute to the enrichment of infective oocysts and lead to cross-contaminations from one batch to another in washing tank. The inefficacy of chlorine to inactivate *C. parvum* oocysts raises questions about the management of industrial washing to limit cross-contaminations.

## 5. Conclusion

This is the first study which shows the ability of *Cryptosporidium* oocysts to persist and survive on the leaves of lamb's lettuce during plant growth. The washing process applied to MPV has a limited efficiency in removing parasites inoculated onto surface leaves. As expected, chlorine, which is used in some countries as a disinfectant to maintain the bacteriological quality of washing water, has no effect on oocysts. This work suggests that if high contamination occurs during the early stage of lamb's lettuce growth, MPV produce could be contaminated by infective oocysts. Besides, re-using washing water can contribute to enriching washing tanks with infective oocysts leading to cross-contamination between batches. Hence, this work reinforces the relevance of including *Cryptosporidium parvum* hazard management in Good Agricultural Practices (GAPs) and the need for growers to take measures to mitigate and control it during the primary production of leafy green vegetables.

## CRediT authorship contribution statement

Conceptualisation: S.L.C., L.F., I.V. and R.R.; methodology: S.K., C. C.; validation: S.L.C., L.F., R.R. and D.C.; investigation: S.K., C.C.; writing-original draft preparation: S.K.; visualisation: S.L.C., R.R, L.F., I. V., C.C. and D.C.; supervision: D.C., S.L.C., L.F. and R.R.; funding acquisition: L.F., I.V. and S.L.C. All authors have read and agreed to the published version of the manuscript.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Data availability

Data will be made available on request.

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## Appendix

**Table 1**
The chemicals and reagents used in this study. N.A. = not available.

| Chemical | Case-no. | Purity | Supplier |
|---|---|---|---|
| Ascorbic acid | 50–81-7 | 99 % | Sigma, France |
| Calcium hypochlorite, 5 % active chlorine AcrosOrganics | 7681-52-9 | 4.735 % | Thermo Fisher Scientific, Denmark |
| Glucose | 50-99-7 | ≥99.5 % | Sigma, France |
| Glycine | 56-40-6 | ≥99 % | Sigma, France |
| HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) | 7365-45-9 | ≥99.5 % | Sigma, France |
| Insulin | 11061-68-0 | N.A. | Sigma, France |
| Para-aminobenzoic acid | 150-13-0 | ≥99 % | Sigma, France |

| Reagent | Reference | Supplier |
|---|---|---|
| Crypto-Cel FITC stain | Z1RP1 | TCS Biosciences, UK |
| Isolate for IMS of *Cryptosporidium* oocysts | ZAICS100 | TCS Biosciences, UK |
| PBS Gibco™ | 10010-023 | Thermo Fisher Scientific, France |
| Penicillin-streptomycin Corning™ | 15313671 | Thermo Fisher Scientific, France |
| QIAmp DNA Mini kit | 51306 | Qiagen, France |
| RPMI 1640 medium Gibco™ | 11875-093 | Thermo Fisher Scientific, France |

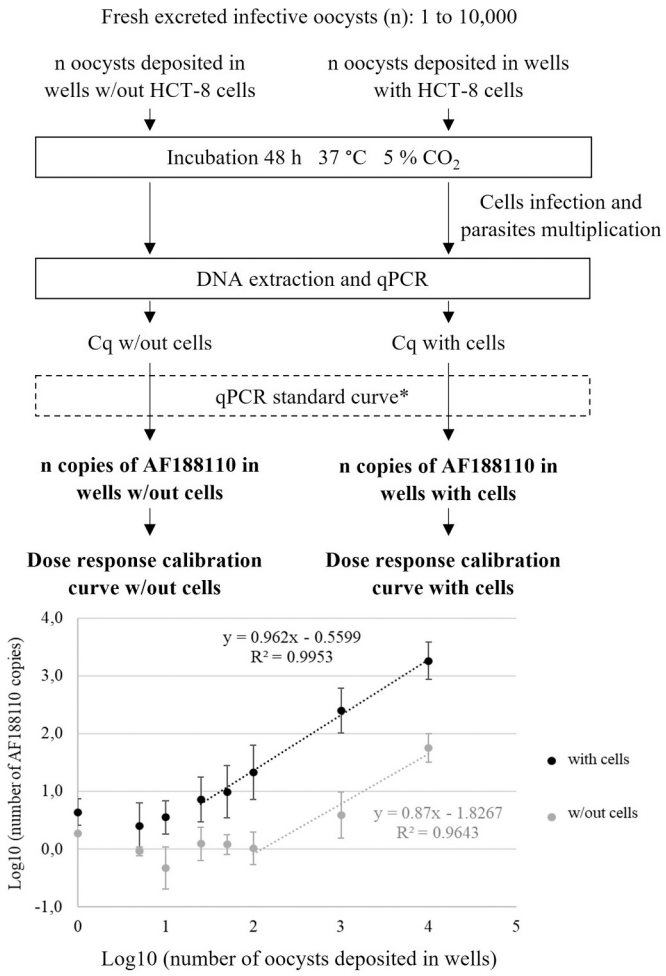


**Fig. 1.** Establishment of dose-response calibration curves. *Cq w/out and with cells are expressed in n copies of AF188110 using the qPCR standard curve, Cq = f (Log10(Af188110 copies/reaction)), with AF188110 copies corresponding to copies of a synthetic dsDNA. Then dose-response calibration curves are established in wells w/out and with cells by plotting the Log10 (number of AF188110 copies) to the Log10 (number of infective oocysts deposited in wells with cells) (black dots) and without cells) (grey dots)). w/out: without.

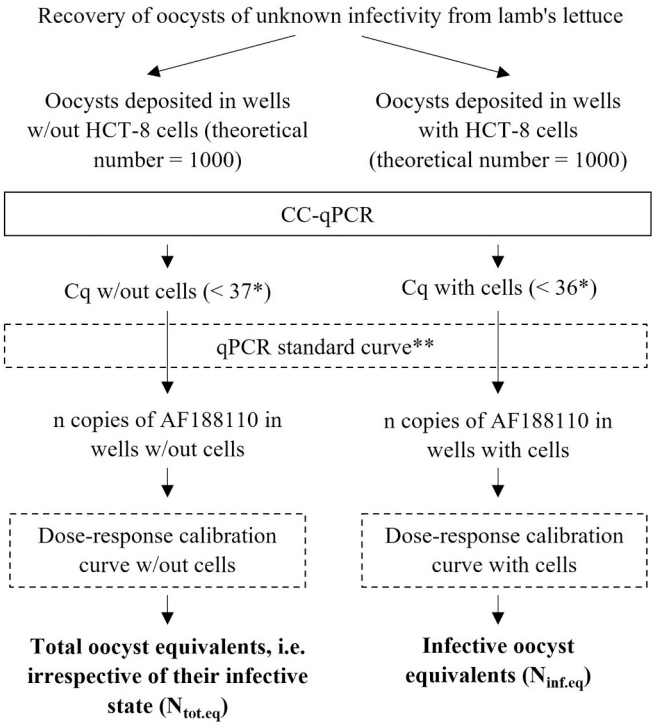


**Fig. 2.** General procedure for quantification of oocysts in lamb's lettuce samples by CC-qPCR. *Cq corresponding to the limit of the quantification of the CC-qPCR assay on lamb's lettuce (as defined by Kubina et al., 2021). **Cq w/out and with cells are expressed in n copies of AF188110 using the qPCR standard curve, Cq = f (Log10(AF188110 copies/reaction)), with AF188110 copies corresponding to copies of a synthetic dsDNA. Then the numbers of copies in wells w/out and with cells are transformed in total oocyst or infective oocyst equivalents respectively, using the corresponding calibration curves. w/out: without.

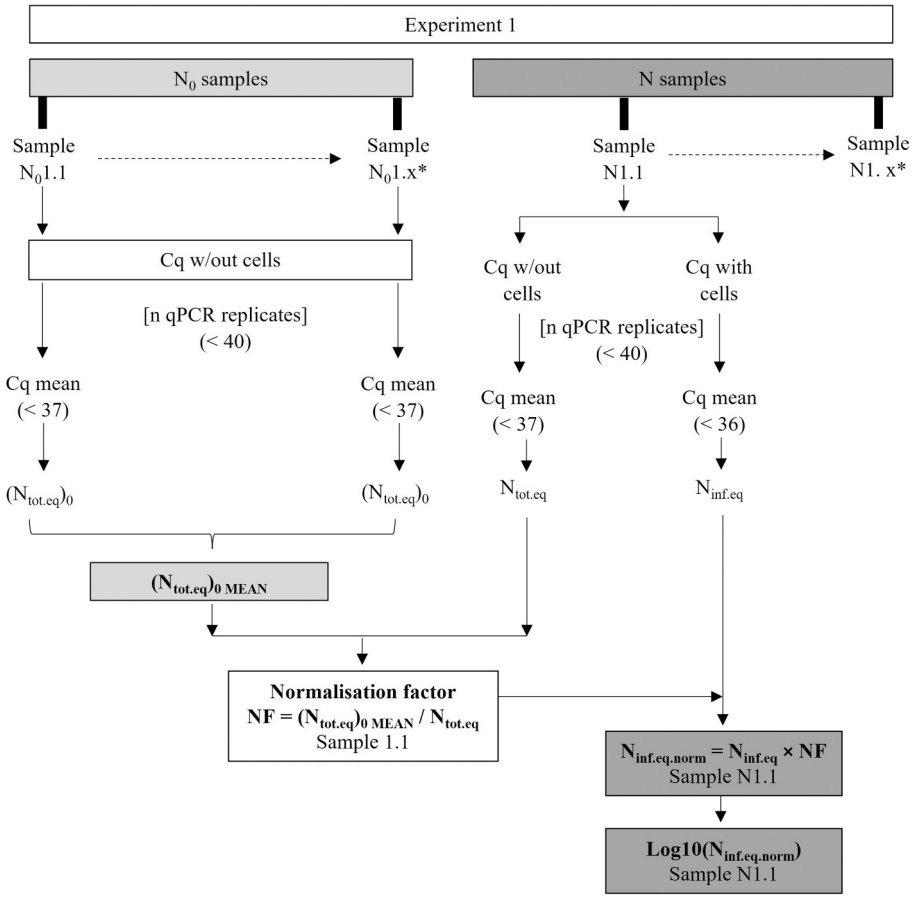


**Fig. 3.** Procedure to normalise the numbers of infective oocyst equivalents as determined by CC-qPCR: example for one sample N (day of plant growth or after the washing process) of one independent experiment. *x = 2 to 6 samples per independent experiment (3 to 4 independent experiments were performed). w/out: without. $N_{tot.eq}$: N total oocyst equivalents. $N_{inf.eq}$: N infective oocyst equivalents. $N_{inf.eq.norm}$: N normalised infective oocyst equivalents.

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